International Journal of Genetic Engineering and Biotechnology.
ISSN 0974-3073 Volume 2, Number 1 (2011), pp. 115-124

http://www.irphouse.com

# An Enzymatic Tablet Method for Quantification of Monocrotophos from Environmental Samples

**Uma Maheswara Rao Ganguru[1]**
**and Yalavarthy Prameela Devi[2]**

*[1]Center for Biotechnology, Institute of Science and Technology,*
*Jawaharlal Nehru Technological University, Hyderabad 500085, India*
*[2]Environmental Biology Laboratory, Department of Zoology,*
*Kakatiya University, Warangal 506009, India*

## Abstract

Monocrotophos is an organophosphorus pesticide, generally used against pest to protect economically impotent crops. Contamination possibility may occur during manufacturing, transport and usage, because of high toxic effects of this pesticide, it is recommended to monitor on the site to prevent advert effects. In the present study, we developed a simple, low cost, viable and sensitive enzymatic tablet method for quantification of monocrotophos from environmental samples. This technique works based on inhibition of the enzyme, succinate dehydrogenase (SDH) (EC.No. 1.3.5.1). The enzyme (SDH) particularly binds to the substrate (sodium succine) present in the sample and develops pink colour in the presence of chromogenic reagent which contains INT (2-(4- Ido-phenyl)-3-(4-nitrophenyl)-5 phenyl tetrazolium chloride) and PMS (N-methyl phenazonium methosulphate). Egg albumin lyophilized powder was used as the source of SDH enzyme. Two tablets were prepared, one containing the enzyme and the other containing a mixture of substrate and chromogenic agent. Tablet method was optimized by colorimetric method: optimum temperature (80ºC), enzyme concentration (10 mg/tablet) and optimum time (30 min). Percentage of inhibition and concentration of monocrotophos at a range of 0-110 µg was plotted, which was observed to follow the Beer Lambert's Law. A colour chart has been prepared for quantification of monocrotophos based on the formation of formazan in the reaction. If the concentration of monocrotophos in environmental samples is within the range, the samples can be quantified by comparing with the colour chart. The method is successfully applied for quantification of monocrotophos from environmental samples.

**Keywords:** Monocrotophos, SDH, tablet, quantification, environment.

## Introduction

Monocrotophos is a systemic broad spectrum organophosphorus insecticide, widely used against pests to protect economically important crops, such as cotton and chilly (Lee et al. 1990; Tomlin, 1994). It is extreme water soluble. Therefore, it may appear in wastewater generate from manufacturing units. It may remain as residue when sprayed on crops and also enter into surface and ground water through leaching from soil (Tomlin, 1995). Monocrotophos inhibits acetylcholinesterase (AChE) which is an essential enzyme for normal nerve impulse transmission and also affects mainly on organs including skin, eyes and central nervous system. Monocrotophos has the ability to cause chromosomal damage to mammalian cells (Kalyan et al. 2009; Paulo et al. 1996). Residues of monocrotophos from environmental samples can be determined by various instrumental methods like HPLC, HPLC-MS, GC and GC-MS (Donnelly et al. 1900). Using of these methods are non-compact, lengthy and costly. The purpose of the present study is to develop a simple, sensitive, inexpensive enzymatic tablet method for quantification of monocrotophos from environmental samples.

## Materials and Methods

### Monocrotophos & other materials

Monocrotophos (IUPAC Name: Dimethyl (E) -1-methyl-2-methyl-2-(methylcarbamoyl) vinylphosphate) (98% pure), obtained from HYDERABAD CHEMICALS, Hyderabad, India. Fresh chick eggs were procured from MRCB, Hyderabad, India. Sodium succinate, INT (2-(4- Ido-phenyl)-3-(4-nitrophenyl)-5 phenyl tetrazolium chloride) and PMS (N-methyl phenazonium methosulphate), lactose (β-D-galactopyranosyl-(1→4)-D-glucose), insoluble starch, magnesium stearate (magnesium octadecanoate) and SDS (sodium dodecyl sulfate) were purchased from HIMEDIA. The solvent hexane was procured from MERCK.

### Enzyme source

Egg albumin was extracted from fresh egg and an emulsion was prepared at 20% (v/v) concentration in distilled water. This emulsion was lyophilized and the powder was directly used as a source of succinate dehydrogenase (SDH) (EC.No. 1.3.5.1) enzyme.

### Preparation of tablets

Two tablets (i.e., tablet-A and tablet-B) were prepared. As given in Table 1, tablet A was prepared with lyophilized egg albumin powder. Tablet B was prepared with sodium succinate (substrate) and INT & PMS mixture (chromogenic agents) (Table 2). The above compositions for tablet A and B were separately mixed in a china dish and then placed in the cavity of the tablet machine for tablet preparation by direct compression method.

**Table 1:** Composition of Tablet A.

| Component | Concentration (mg/tablet) |
|---|---|
| Enzyme powder | 10 |
| Lactose | 335 |
| Starch (in soluble) | 50 |
| Magnesium stearate | 5 |
| Total: | 400 mg |

**Optimization of tablet method**

The tablet method was standardized by colorimetric method. The tablets, A and B were separately dissolved in 20 mL and 10 mL of distilled water respectively. As given in Table. 3, 1.0 mL of the reaction mixture was prepared and incubated at various conditions to optimize the assay i.e. incubation temperature 10-100˚C, time 0-60 minutes and enzyme concentration 0-20 mg/per tablet. The enzyme reaction was stopped by addition of 2 mL of 1% SDS and then the optical density was measured at 495 nm using a colorimeter.

**Table 2:** Composition of Tablet B.

| Component | Concentration (mg/tablet) |
|---|---|
| INT | 8 |
| PMS | 3 |
| Sodium succinate | 4 |
| Lactose | 330 |
| Starch (in soluble) | 50 |
| Magnesium stearate | 5 |
| Total: | 400 mg |

**Calibration curve and color chart**

Standard solutions of monocrotophos were prepared to assess the inhibition of SDH enzyme activity. 1ml reaction mixture was prepared (Table 3) and incubated at 80ºC for 30 min. The reaction was stopped using 1% SDS, and then the optical density was measured at 495 nm with the help of a colorimeter. The control was prepared with the same reaction mixture which contained 0.2 mL of distilled water instead of monocrotophos standard. Standard graph was prepared based on the amount of pink colour formazan formed due to the SDH activity (OD vs. formazan). Color chart was also developed for quantification of monocrotophos by the color thickness of the standards using M.S. word 2007 font color.

**Table 3:** Composition of reaction mixture.

| | |
|---|---|
| Tablet - A (Enzyme) | 0.4 ml |
| Monocrotophos standard | 0.2 ml |
| Incubation for 10 minutes at 37□C | |
| Tablet – B (Substrate) | 0.2 ml |
| Distilled water | 0.2 ml |
| Total: | 1 ml |

**Quantification of monocrotophos from environmental samples**

The present work was undertaken to assess the applicability of the developed tablet method for quantification of monocrotophos from environmental samples. Water and soil samples were collected from the industrial and agricultural areas of Balanagar and Jeedimetla, Hyderabad, INDIA. All the samples were collected in fresh polypropylene bags and were analyzed within 4 hours of collection. The collected water samples were filtered through whatman No. 40 filter paper. Filtrate (1 liter) was extracted into hexane and further concentrated using rota vapour at room temperature (37□C). After complete removal of hexane, the final content was dissolved in 1 mL of distilled water. Soil samples (50 gm) were ground separately and extracted with 100 mL of distilled water. This water was separated from soil and concentrated by the above mentioned procedure. Then the final content was dissolved in 1 mL of distilled water for evaluating the content of monocrotophos. The samples were analyzed by the developed enzymatic method and also compared with GC-MS to know the performance of developed tablet method.

**Percent SDH inhibition (I%)**

The percent SDH inhibition was calculated by the following formula which is based on the formazan formation and it's optical density at 495nm.

$$\textbf{Percent inhibition} = \frac{\textbf{C - E}}{\textbf{C}} \textbf{ X 100}$$

C = optical density of control
E = optical density of sample

**Table 4:** Quantification of environmental water and soil samples for the presence of monocrotophos.

| S.No. | Sample | Quantification by tablet method (ppm) | Quantification by GC/MS (ppm) |
|---|---|---|---|
| Water Samples | | | |
| 1. | Agricultural waste water | - ve | - ve |
| 2. | Balanagar pesticide industrial waste water | 0.05 | 0.048 ± 0.002 |

| | | | |
|---|---|---|---|
| 3. | Jeedimetla pesticide industrial waste water | 0.02 | 0.022 ± 0.006 |
| Soil Samples | | | |
| 1. | Agricultural land soil | - ve | - ve |
| 2. | Balanagar pesticide industrial area soil | - ve | - ve |
| 3. | Jeedimetla pesticide industrial area soil | - ve | - ve |

± S.D of mean of 4 observations.

## Results and Discussion

### Basic principle

The basic principle involved in quantification of monocrotophos is based on the biochemical reaction between monocrotophos and enzyme SDH. SDH is a member of citric acid cycle which catalyses the oxidation of succinate to fumarate [Michele et al., 2004]. SDH activity can be assessed by the reduction of tetrazolium salts to deeply colored water insoluble formazan in the presence of substrate (sodium succinate) [Defendi et al., 1995; Glick and Nayyar, 1956; Kun and Ahood, 1949]. In the presence of monocrotophos (inhibitor), this enzyme reaction is inhibited. This inhibitory nature was made use for quantification of monocrotophos. In the present experiment INT was employed as a tetrazolium salt and PMS was used as an exogenous electron carrier to speed up the reaction process.

### Optimum conditions

The optimum temperature was found at 80□C (Figure 1). In general, this temperature is very high when compared to the growing temperature of chick. The incubation time for maximum activity of SDH was 30 min and no significant activity was observed ahead of 30 min (Figure 1). Generally, the concentration of substrate influences enzyme inhibition. Enzyme inhibition increases with increase in substrate concentration [Kok et al., 2002]. However, in case of SDH assay sodium succinate is not a rate limiting factor [Chandra, 1999]. Hence, in the present study, appropriate concentration of the substrate was used. The maximum enzyme activity was obtained at the concentration of 10 mg/tablet and enhanced concentration of enzyme did not produced in significant increase of activity. In the present experiment, tablet A was made with 10 mg of lyophilized egg albumin powder and which is sufficient for analyzing 50 samples.

However, the enzyme sensitivity was found to increase in the presence of inhibitor when used in lower concentrations of the enzyme [Sofia and Nikos, 2005; Sofia et al., 2005; Shan et al., 2004; Mohammadi et al., 2005]. Experiments of detection and determination of inhibitors (pesticides and heavy metals) are generally performed in aqueous solutions. Some enzymes work strongly, when experiments are conducted in organic solvents [Amine et al 2004]. Andreescu et al., reported the detection of

pesticides dichlorvos, diazinon and fenthion in the presence of ethanol using an immobilized acetyl cholinesterase [Andreescu et al., 2002]. However, monocrotophos is extreme water soluble, so the whole experiments were conducted in aqueous solution.

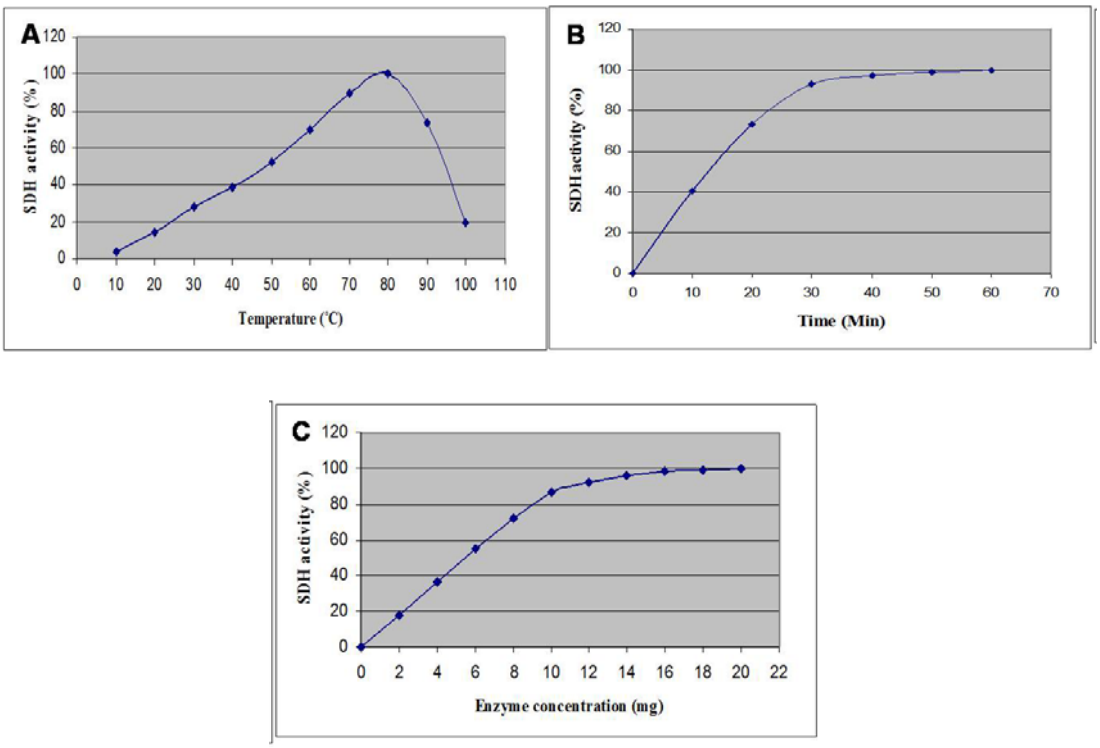


**Figure 1:** Profile for standardization of tablet method for egg albumin SDH enzyme. Standardization of temperature (A), Time (B) and Enzyme concentration (C).

**Standard graph and color chart**

Standard graph was plotted to analyze the samples in the range of 0-110 µg based on the % SDH inhibition. As given in Figure 2, the % enzyme inhibition increased with increasing the concentration of monocrotophos. Typically, the limit of detection (LOD) is based on the concentration of inhibitor [Azizet al., 2006]. On the other hand, the detection limit also depends on the incubation time of the enzyme and inhibitor [Kuswandi, 2003]. A study was conducted with incubation time of 30 min for the detection of paraoxan with 10 mg detection limit [Ciucu et al., 2003]. Kok et al., performed a study to know the residual enzymatic activity after incubation with inhibitor using different incubation times (5, 15, 30 min) in the presence of AChE and ChO bienzymatic system [Kok et al., 2002]. The materials used as support matrices may also inhibit the enzyme activity. However, in the experiment the materials used for the making the tablet A and B cannot inhibit the enzyme SDH [Chandra, 1999]. As shown in Figure 3, a colour chart was prepared. It is showing the gradation of

color with increased inhibition at various concentrations of monocrotophos. This chart help to match the color after the reaction process is over and by this color comparison, the concentration of monocrotophos in the sample can be analyzed. This chart is useful only if the concentration of sample is within the range of 0-110 µg. The pink color formazan produced is inversely proportional to the concentration of monocrotophos. In case of higher monocrotophos levels, samples can be diluted accordingly for bringing them to the quantification limit for further analysis. Borosil reaction test tube (12x75 mm) was used to prepare the colour chart. This information has to be noted, since the transmission of colour intensity varies with size and types of the glass tube and hence advised to use the same.

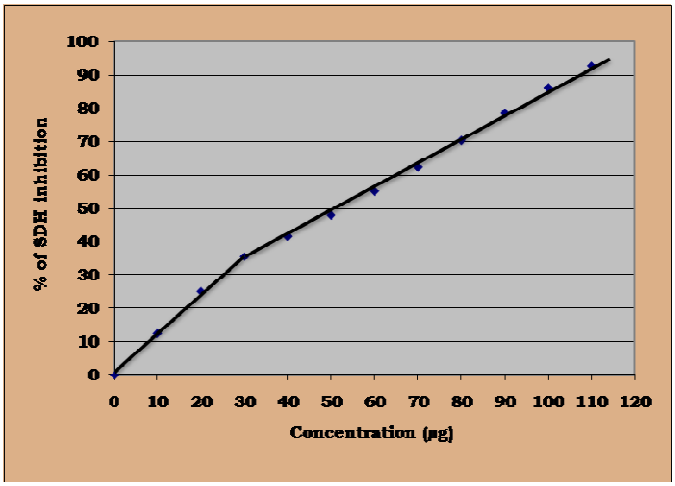


**Figure 2:** Calibration curve showing SDH inhibition and concentration of monocrotophos.

**Analysis of monocrotophos from environmental samples**

The developed tablet method was successfully employed in testing the amount of monocrotophos from environmental samples. The environmental samples were also analyzed by standard instrumental method GC-MS to know the performance of the developed tablet method in quantification of monocrotophos. The concentration of monocrotophos in Balanagar pesticide industrial waste water was found to 0.05 ppm by developed tablet method, whereas it was found to be 0.048ppm by GC-MS. In case of Jeedimetla pesticide industrial waste water, the monocrotophos concentration was found at 0.02 ppm and 0.22 ppm by the developed tablet method and GC-MS respectively. However, there was no monocrotophos found in Agricultural waste water sample and all other soil samples by both the methods (Table 4.).

The developed method is quite useful as it can also be employed in the field. As, the SDH enzyme may be inhibited by a number of pollutants, so it is advised to know the $R_f$ value of monocrotophos for confirmation by TLC.

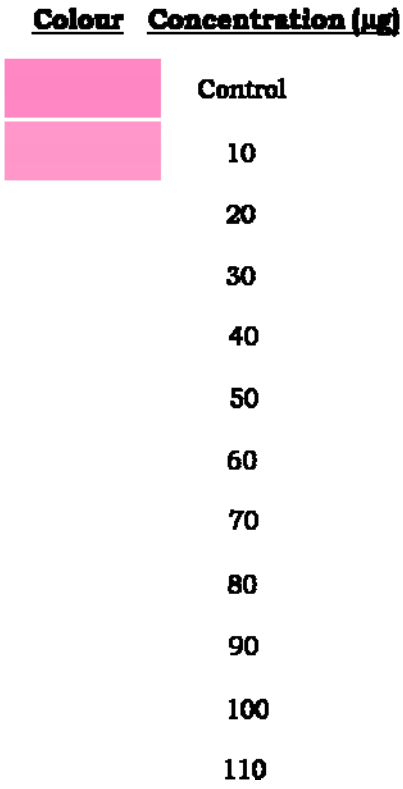


**Figure 3:** Gradation of formazan showing at various concentrations of monocrotophos after SDH inhibition.

## Conclusion

The enzymatic method developed in the present study is very useful for simple, quick and timely monitoring of monocrotophos from environmental samples where the other methods (sophisticated equipments) are time consuming, expensive and unsuitable for field use.

## Acknowledgements

The authors acknowledge UGC, INDIA for the financial support offered to this project and thank to JNTUH authorities for providing laboratory facilities in undertaking this research work.

## References

[1] Sofia, S. and Nikos. A. C. 2005. Lowering the detection limit of the acetyl cholinesterase biosensor using a nanoporous carbon matrix. J. Analytica chemica Acta. 530, pp. 199-204.

[2] Sofia, S., Dider, F. and Nikos, A. C., 2005. Genetically engineered acetyl cholinesterase based biosensors for attomolar detection of DDVP. Biosens. Bioelectron. 20, pp. 2347-2352.
[3] Michele, S., Paola, C. B., Ilaria, D. M., Tiziana, L. and Claudio, F. 2004. The Deletion of the Succinate Dehydrogenase Gene KlSDH1 in Kluyveromyces lactis Does Not Lead to Respiratory Deficiency, J. Eukaryotic Cell. 3, pp. 589-597.
[4] Defendi, V. and Pearson B. 1995. Quantitative estimation of succinic dehydrogenase activity in a single microscopic tissue section. J Histochem Cytochem. 3, pp. 61-69.
[5] Glick, D. and Nayyar, SN. 1956. Studies in histochemistry, XLII. Further studies on the determination of succinic dehydrogenase in microgram amounts of tissue and distribution of the activity in the bovine adrenal. J Histochem Cytochem. 4, pp. 389-396.
[6] Kun, E. and Ahood, L.G. 1949. Colorimetric estimation of succinic dehydrogenase by triphenyltetrazolium chloride. Science. 109 (1949), pp. 144-146.
[7] Kok, F.N., Bozoglu, F. and Hasirci, V. 2002. Construction of an acethylcholinesterase-choline oxidase biosensor for aldicarb determination. Biosens. Bioelectron. 17, pp. 531-539.
[8] Chandra Mouli, G. V. 1999. Development of tablet method for the determination of heavy metals from environmental samples. Ph.D. thesis. Kakatiya University.
[9] Shan, D., Mousty, C. and Cosnier, S. 2004. Subnanomolar cyanide detection at polyphenol oxidase/clay biosensors. Anal. Chem. 76, pp. 178-183.
[10] Mohammadi, H., Amine, A., Cosnier, S. and Mousty, C. 2005. Mercury enzyme inhibition assays with an amperometric sucrose biosensor based on a trienzymatic–clay matrix. Anal. Chem. Acta. 543, pp. 143-149.
[11] Amine, A., Mohammadi, H., Arduini., F., Ricci, F., Moscone, D. and Palleschi, G. 2004. Extraction of enzyme inhibitors using a mixture of organic solvent and aqueous solution and their detection with electrochemical biosensors, Eighth World Congress on Biosensors, Granada in Spain.
[12] Andreescu, S., Noguer, T., Magearu, V. and Marty, J. L. 2002. Screenprinted electrode based on AChE for the detection of pesticides in presence of organic solvents. Talanta. 57, pp. 169-176.
[13] Aziz, A., Hasna, M., Ilhame, B. and Giuseppe, P. 2006. Enzyme inhibition-based biosensors for food safety and environmental monitoring, Biosens and Bioelec. 21, pp. 1405-1423.
[14] Kuswandi, B. 2003. Simple optical fibre biosensor based on immobilized enzyme for monitoring of trace heavy metal ions. Anal. Bioanal. Chem. 376, pp. 1104-1110.
[15] Ciucu, A., Negulescu, C. and Baldwin, R.P. 2003. Detection of pesticides using an amperometric biosensor based on ferophthalocyanine chemically modified carbon paste electrode and immobilized bienzymatic system. Biosens. Bioelectron. 18, pp. 303-310.

[16] Lee, P. W., Fukuto, J. M., Hernandez, H. and Stearns, S. M. 1990. Fate of monocrotophos in the environment. Journal of Agricultural Food Chemistry. 38(2), pp. 567-573.

[17] Tomlin, C. (ed), 1994. The Pesticide Manual, 10th Edition, Crop Protection Publications, British Crop Protection Council and Royal Society of Chemistry.

[18] Tomlin, C. (ed), 1995. The Pesticide Manual (Incorporating the agrochemicals handbook). 10th Edition, British Crop Protection Council.

[19] Kalyan, Chakravarthi, B., Rambabu, Naravaneni., Philip, G. H., Srinivasulu Redddy, C. 2009. Investigation of monocrotophos toxic effects on human lymphocytes at cytogenetic level. African Journal of Biotechnology. 8(10), pp. 2042-2046.

[20] Paulo, Peiti, Jr., Elza, T, Sakamoto., Hojo, Iice, M. and De Syllos, Colus. 1996. Genotoxic activity of the insecticide Nuvacron (Monocrotophos) detected by the micronucleus test in bone marrow erytrocytes of mice and in CHO cells. Brazilian Journal of Genetics. 19(4), pp. 571-576.

[21] Donnelly, Betowski, L. and Tammy, L, Jones. 1900. Analysis of Organophosphorus Pesticide Samples by High-Performance Liquid Chromatography/Mass Spectrometry and High-Performance Liquid Chromatography/Mass Spectrometry/Mass Spectrometry. Environ. Sci. Technol. 22, pp. 1430-1434.